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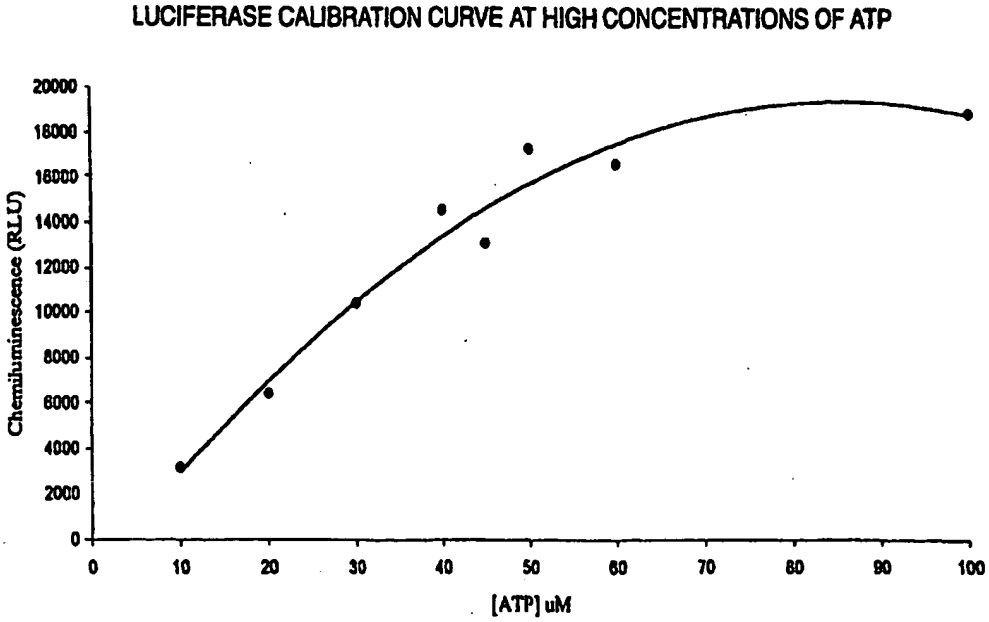

(57) Abstract

The invention provides a method of monitoring the bacterial contamination of a wound comprising monitoring the adenosine triphosphate (ATP) concentration of wound fluid removed from the wound. Preferably, an enzyme–coupled reaction such as the luciferin–luciferase reaction is used for the monitoring. Diagnostic kits and wound dressings adapted for use in the method are also provided.

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METHOD OF MONITORING BACTERIAL CONTAMINATION OF A WOUND

The present invention relates to a method of monitoring the bacterial contamination of a wound by monitoring the ATP concentration of wound fluid or exudate. The present invention also relates to devices and kits for use in such methods.

In mammals, injury triggers an organised complex cascade of cellular and biochemical events that result in a healed wound. Wound healing is a complex dynamic process that results in the restoration of anatomic continuity and function; an ideally healed wound is one that has returned to normal anatomic structure, function and appearance.

Infection of wounds by bacteria delays the healing process, since bacteria compete for nutrients and oxygen with macrophages and fibroblasts, whose activity are essential for the healing of the wound. Infection results when bacteria achieve dominance over the systemic and local factors of host resistance. Infection is therefore a manifestation of a disturbed host/bacteria equilibrium in favour of the invading bacteria. This elicits a systemic septic response, and also inhibits the multiple processes involved in wound healing. Lastly, infection can result in a prolonged inflammatory phase and thus slow healing, or may cause further necrosis of the wound. The granulation phase of the healing process will begin only after the infection has subsided.

The persistent presence of bacteria in injured tissue results in the prolonged elevation of proinflammatory cytokines such as interleukin-1 and tumour necrosis factor alpha (TNF-α). This in turn causes increases in the levels of matrix metalloproteinases, a decreased level of tissue inhibitors to the metalloproteinases (TIMP), and a decreased production of growth factors.

Chronically contaminated wounds all contain a tissue bacterial flora (see, for example, Miller and Gilchrist, "Understanding wound cleaning and infection", *Professional Nurse* 1998, published by MacMillan Magazines Ltd.). These bacteria may be indigenous to the patient or might be exogenous to the wound. Closure, or eventual healing of the wound is often based on a physician's ability to control the level of this bacterial flora.

Current methods used to identify bacterial infection rely mainly on judgement of the odour and appearance of a wound. With experience, it is possible to identify an infection in a wound by certain chemical signs such as redness or pain. Some clinicians take swabs that are then cultured in the laboratory to identify specific organisms, but this technique takes time.

There is thus a long felt need for a diagnostic aid that would assist in the monitoring of the bacterial contamination of a wound. Such a diagnostic would enable small-scale bacterial infection to be treated before wound chronicity sets in and would also allow the monitoring of an infected wound to assess the success of an anti-bacterial treatment.

It has been discovered that the adenosine triphosphate (ATP) concentration of a wound correlates closely with the level of bacterial contamination. According to the present invention, there is therefore provided a method of monitoring the bacterial contamination of a wound comprising monitoring the ATP concentration of wound fluid. The measurement of the ATP concentration of the wound fluid allows the level of bacterial contamination to be accurately assessed. The step of monitoring is preferably carried out on wound fluid that has been removed from the body of the patient, but can also be performed on wound fluid *in situ*.

Any type of wound may be diagnosed for infection according to the method of the present invention. For example, the wound may be an acute wound such as an acute traumatic laceration, perhaps resulting from an intentional operative incision, or the wound may be a chronic wound. The method of the invention is envisaged as being most useful in the diagnosis of bacterial contamination of a chronic wound. Preferably, the chronic wound is selected from the group consisting of venous ulcers, pressure sores, decubitis ulcers, diabetic ulcers and chronic ulcers of unknown aetiology.

According to the method of the present invention, the diagnostic assay is designed so as to provide a correlation between a measurable signal and the size of the bacterial population that is associated with clinical infection. Under present clinical standards, bacterial infection of wounds is generally taken to be 10^5cfu/cm^2. Accordingly, the system should be set up so that a detectable response is tripped at around 10^4cfu/cm^2 bacteria so as to give a warning of bacterial contamination. The ATP concentration correlated with such a level of bacteria is far in excess of the normal ATP turnover in

the wound; accordingly, background ATP concentrations do not distort the signal to a significant extent.

As used herein, the term wound fluid is meant to refer to the exudate that is secreted or discharged by cells in the environment of the wound. This fluid contains cells, both living and dead, and a variety of inflammatory cytokines.

By ATP concentration is meant the free concentration of ATP in the wound fluid. The ATP concentration may be assessed *in situ*, or alternatively a sample of wound fluid may be taken as a clinical swab or as a fluid sample.

The ATP concentration of the wound fluid may be monitored by any method known to those of skill in the art. Suitable methods include those utilising chemical or enzyme-linked reactions, or by conjugating the reaction of ATP to immunological, fluorogenic, chemiluminescent, chromogenic or radioactive detection mechanisms.

Reactions in which ATP participates as a substrate are usually driven in the direction leading to hydrolysis of ATP. The chemical energy released from hydrolysis is then utilised under physiological conditions in active transport, or may be converted to mechanical, light or electrical energy, or may be released as heat. Accordingly, any chemical reaction to which ATP concentration can be stoichiometrically coupled may be used in the method of the present invention.

It is envisaged that the most convenient method of ATP detection will involve the use of enzymes whose reaction with substrate is driven by ATP and which either themselves, or through coupled reactions give a detectable signal that is proportionate to ATP levels. Preferably, ATP concentration is coupled to the generation of a detectable signal through the action of enzymes that dissipate the energy of ATP hydrolysis as light or electrochemical energy. Suitable enzymatic reactions include, but are not limited to the luciferase-luciferin reaction, the hydrolysis of ATP by alkaline phosphatase.

Alkaline phosphatase (ALP) is an enzyme that catalyses the hydrolysis of orthophosphoric monoester to an alcohol and orthophosphate. ATP is a substrate of this enzyme. There are many commercially available ALP assays that couple the activity of this enzyme to ATP concentration by electrochemical modulation. For example, the

well-known electrochemical luminescence (ECL) detection method (Amersham) may be used to couple the ALP-catalysed hydrolysis of ATP to give a measurable signal that is proportionate to the levels of ATP in wound fluid. The "Alkphos direct" labelling and detection systems (Amersham Life Sciences) are of particular usefulness in this regard.

The method of the present invention may also utilise the luciferase-luciferin reaction. This assay is particularly applicable to the method of the present invention due to its simplicity and sensitivity. The luciferase enzyme catalyses reactions in which a singlet intermediate is formed in high quantum yield. This intermediate decays, simultaneously omitting visible light. In the presence of luciferin (D-LH_2), ATP-Mg^{2+}, and molecular oxygen, luciferase catalyses the production of light according to the following reactions determined by DeLuca *et al.* Method Enzymol., vol. **LVII**, pages 3-15 (1978);

$$Mg_{2+} + \Sigma$$

$$D - LH_2 + ATP \Leftrightarrow E.LH_2AMP + PPi$$

$$E.LH_2AMP + O_2 \Rightarrow E.P + AMP + CO_2 + LIGHT$$

The first reaction involves the activation of luciferin and hydrolysis of ATP resulting in the formation of an enzyme-bound luciferyl adenylate ($E.LH_2AMP$). The second reaction requires molecular oxygen and leads to the formation of enzyme-bound excited product, which subsequently decomposes to give off light and enzyme-bound product. Quantitative liberation of CO_2 from the luciferin during the reaction is also observed.

Coenzyme A may be used as a co-factor in this reaction; in the presence of coenzyme A, oxidation occurs from luciferyl-CoA with more favourable total kinetics, resulting in the generation of constant light intensity that is proportionate to the ATP concentration.

In order to measure the generation of light, a luminometer may be used. However, it will be apparent to the skilled artisan that this assay is easily adaptable to measurement of light generation in scintillation counters or using photographic film.

To allow measurement of the ATP concentration in a wound, a sample of wound fluid must be added to the ATP assay system. Measurement may either be made *in situ*, or

fluid may be removed from the wound for subsequent ATP measurement. The decision as to which method is used will depend upon the type of wound in question.

For example, in the case of surface-exposed wounds, a clinical swab, dressing, "dipstick" or other biosensor device may be applied directly to the surface of the wound. This device should contain all of the required components of the ATP-linked reaction so that the reaction itself may proceed *in situ*. The device can then be removed from the wound and the signal measured by the appropriate means. In many cases, a physician may not actually require an accurate assessment of the precise degree of bacterial infection, but may just wish to know whether there is a sufficient degree of infection to warrant prophylactic action. In these cases, visible assessment of the dressing may be sufficient to allow identification of the specific areas of infection. Unnecessary treatment of healthy granulating tissue can then be avoided.

A dressing that allows mapping of the infected areas of a wound will be preferable in certain instances. Diagnostic wound mapping sheets that could be adapted to the methods of the present invention are described in co-pending application GB 9705081.9 filed on 12th March 1997, the entire content of which is hereby incorporated by reference.

Immobilisation of reaction components onto a dipstick, wound mapping sheet or other solid or gel substrate offers the opportunity of performing a more quantitative measurement. For example, in the case of a reaction linked to the generation of a colour, or of light, the device may be transferred to a spectrometer, a luminometer or a scintillation counter. Suitable methods of analysis will be apparent to those of skill in the art.

Immobilisation of the reaction components to a small biosensor device will also have the advantage that less of the components (such as enzyme and substrate) are needed. The device will thus be less expensive to manufacture than a dressing that needs to have a large surface area in order to allow the mapping of a large wound area.

Methods for the incorporation of the components of the assay reaction onto a clinical dressing, "dipstick", sheet or other biosensor are routine in the art. See for example Fägerstam and Karlsson (1994) *Immunochemistry*, 949-970.

The ATP concentration of a wound may alternatively be measured in an aqueous assay system. Wound fluid may be extracted directly from the environment of the wound or can be washed off the wound using a saline buffer. The resulting solution can then be assayed for ATP concentration in a test tube or in a microassay plate. Clearly, this will allow a more accurate assessment of the exact levels of bacteria in a wound.

Such a method will be preferable for use in cases in which the wound is too small or too inaccessible to allow access of a diagnostic device such as a dipstick. This method has the additional advantage that the wound exudate sample may be diluted. Under most diagnosis methods, dilution will still allow accurate assessment of ATP amount, yet will enable an operative to assess the concentration of a wound from which it is not possible to extract more than a few microlitres of exudate. Such wounds will include small or internal ulcers and pressure sores.

It will be clear that an aqueous assay system is more applicable to use in a laboratory environment, whereas a wound dressing containing the necessary reaction components will be more suitable for use in a hospital or domestic environment.

Specific embodiments of the present invention will now be described in more detail, by way of example, with reference to the accompanying drawings, in which:-

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a luciferase calibration curve at high concentrations of ATP.

Figure 2 shows a luciferase calibration curve at low concentrations of ATP.

Figure 3 shows the stability of luciferase at different temperatures.

Figure 4 shows the elucidation of ATP concentration for different amounts of *E. coli* using a luciferase assay.

Figure 5 shows the measurement of ATP concentration for different amounts of *S. aureus* using the Hy-Lite system (Merck & Co.).

Figure 6 shows the measurement of ATP concentration for different amounts of *E. coli* using the Hy-Lite system (Merck & Co.).

Figure 7 shows an ATP standard curve obtained using the Sigma ATP detection kit.

Figure 8 shows an ATP curve obtained using *E. coli* diluted in wound fluid analogue.

Figure 9 shows an ATP curve obtained using *S. aureus* diluted in wound fluid analogue.

Figure 10 shows an ATP standard curve measured in porcine wound fluid.

EXAMPLES

Materials and stock solutions used in Examples 1 and 2

pH 7.8 Luciferase storage buffer

50 ml stock containing:-	0.298 g of 25 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES)
	0.051 g of 5mM $MgCl_2$
	0.077 g of 10 mM dithiothreitol (DTT)
	12.5 µl of Triton X-100 (RTM)
	25 ml of Glycerol

pH 7.8 Luciferase reaction buffer

50 ml stock containing:-	0.298g of 25 mM HEPES
	0.051g of 5 mM $MgCl_2$
	0.077g of 10 mM DTT

Luciferase

- 1ml of 32 µM Luciferase stock:- 2 mg of solid Luciferase (Sigma L9506)
  1 ml of pH 7.8 Luciferase storage buffer
- 1ml of 32 nM Luciferase:- 10 µl of 32 µM Luciferase
  990 µl of pH 7.8 Luciferase storage buffer

Luciferin

- 1ml of 18 mM Luciferin stock:- 5 mg of solid Luciferin (Sigma L6152)
  1 ml of pH 7.8 Spectroscopy buffer
- 1ml of 0.9 mM Luciferin containing:- 50 µl of 18 mM Luciferin stock
  1 ml of pH 7.8 Spectroscopy buffer

pH 7 ATP

- 1 ml of 10 mM ATP containing:- 0.055 g of solid ATP
  10 ml of spectroscopy buffer

Serial dilutions were carried out to product ATP concentrations ranging from 10μM – 100μM to produce a calibration curve with the different ATP concentrations.

***E.coli* $DH_5\alpha$**

E.coli was supplied by the Biochemistry department, from The University of Birmingham

Absorbance 1 = $5x10^8$ cells of bacteria/ml

10 μl of stock; 990 μl sterile water: Absorbance 0.267 = $1.3x10^8$ cells of *E.coli*/ml

E. coli ranged between: 0-20 μl (0 – $2.6x10^8$ cells of *E.coli*/ml)

Example 1: Establishing the optimal conditions for the luciferase assay

A calibration curve was developed for the luciferase assay under optimal conditions. This curve gave information regarding the minimum ATP concentration necessary for detection.

Determination of ATP concentration with maximum absorbance

Maximum absorbance for ATP detected by U.V. spectrophotometer is approximately 0.6 U.

ATP

- 10 mM ATP gives an absorbance of 154,
- 40 μM ATP, by Beer Lambert Law, would give an absorbance of 0.616.
- U.V. spectrophotometer provided a maximum absorbance of a 40 μM ATP in lml of pH 7.8 spectroscopy buffer, in a spectrum scanned between 220 and 300 nm.
- 40 μM ATP provided $A_{260.5}$ of 0.476 units [data not shown].

Luciferin

- 18 mM luciferin gives an absorbance of 347.7.
- 36 μM ATP, by Beer Lambert Law, would give an absorbance of 0.649.
- U.V. spectrophotometer provided a maximum absorbance of 36μM luciferin, in 1 ml of pH 7.8 spectroscopy buffer, spectrum scanned between 250 and 350 nm.
- 36 μM luciferin provided $A_{329.6}$ of 0.573 units [data not shown].

Luciferase Calibration curve

Having achieved the ideal ATP and luciferin concentrations for the assay, the reaction was initiated. A typical assay mixture contained 1 ml pH 7.8 luciferase reaction buffer, 0.32nM luciferase (10μl of 32nM) made in pH 7.8 luciferase storage buffer, and 9μM luciferin (10μl of 0.9mM). Appropriate amounts of ATP ranging between 10^{-2} nM – 10^{4} nM were added to initiate the reaction and record the maximum chemiluminescence.

Figure 1 shows a calibration curve with high ATP concentrations, and confirms a maximum activity at 50 μM ATP. Figure 2 shows lower ATP concentrations, however, this curve also confirms a detection limit of 1nM. The rate of ATP detected is clearly proportional to quantity of ATP.

Stability of luciferase

The stability of the luciferase assay was investigated at different temperatures over a one week period in order to investigate the ideal temperature and length of storage for the assay.

Three aliquots of luciferase at a concentration of 32nM (330 μl) each stored at different temperatures [-20°, 4° and 20°C] were developed to determine the activity of luciferase over a one week period. A maximum chemiluminescence was recorded when 25μl of 25μM ALP (630 nM) was taken at each temperature range and added into 1 ml pH 7.8 luciferase reaction buffer with 9 nM luciferin and 50 μM ATP. The measurements were repeated after storage for a further five days to allow ample time for any modification in luciferase activity.

A concentration of 32nM luciferase was found to be most stable at -20°C confirming a 37% luciferase degradation, over a one week period. Whereas, a 100% luciferase degradation at 20°C, and a 68% luciferase degradation was observed over a one week period [Figure 3].

Example 2: Testing luciferase assays on biological samples

ATP concentrations were investigated in saliva and in an *E. coli* culture, using the formulated assay.

Detection of ATP in saliva and tooth plaque ± extractant

A luciferase assay kit was developed in the addition of 0.32nM luciferase and 9μM luciferin in 1ml pH 7.8 luciferase reaction buffer. Maximum chemiluminiscence was measured by the addition of 10 μl of saliva or tooth plaque dissolved in saliva from healthy individuals.

The same procedure was investigated by the addition of 10 μl of extractant (1% Tween 80) in saliva or tooth plaque (left for approximately 5 minutes), into the luciferase assay.

Approximately 80% increase in ATP detection in saliva was confirmed in the presence of extractant, compared to the absence of extractant. A control was investigated in the substitution of saliva or tooth plaque with sterile water, confirming undetectable ATP [Table 1].

Table 1. Levels of ATP in saliva & tooth plaque.

Sample (10 μl)	ATP (nM)
Saliva	0
Tooth plaque	2.5
Saliva + 10 μl extractant	2.5
Tooth plaque + 10μl extractant	5.0
Saliva + 20μl extractant	2.6
Saliva + 30μl extractant	2.6

Detection of ATP in different concentrations of extractant

A luciferase assay kit was produced from the addition of 0.32 nM luciferase and 9 μM luciferin in 1 ml pH 7.8 luciferase reaction buffer. ATP detection was initiated in the addition of 10 μl of saliva and 10 μl of extractant (left for approximately 5 minutes), into the luciferase assay. The amount of extractant was varied between 10-30 μl.

The addition of 10μl extractant to saliva was found to be suitable to give a maximum ATP detection. An increase in extractant has no relevant significance to the detection [Table 1].

Detection of ATP in *Escherichia coli* ($DH_5\alpha$)

A_{560} was measured for 1/100 dilution of 10 μl *Escherichia coli* to calculate the concentration of the stock solution. A luciferase assay kit was produced in the addition of 0.36 μM luciferase and 9 nM luciferin into 1 ml pH 7.8 luciferase reaction buffer. Maximum chemiluminescence was measured in the presence of 30 μl of extractant to varying amounts of 1.3×10^9 *E.coli*, ranging between 0 – 20 μl, which was inserted in the luciferase assay. The corrected chemiluminescence was read off the calibration curve to determine the amount of ATP in different amounts of *E.coli* under investigation.

The controls involved were:-

- Absence of *E.coli* & presence of extractant,
- Absence of extractant & absence of *E.coli.*

$DH_5\alpha$ *E.coli* (10 μl) has an absorbance of 0.267 at a wavelength of 560nm detected by a U.V. spectrometer.

Absorbance 1 = 5×10^8 cells of bacteria/ml

Absorbance 0.267 = 1.3×10^8 cells of *E.coli*/ml

ATP detection was found to be proportional to the quantity of *E. coli* added to the luciferase assay. 45nM ATP was detected in extractant in the absence of *E. coli* [Figure 4].

Detection of ATP in DH$_5\alpha$*E.coli* in physiological conditions

The above procedure was repeated under physiological conditions in the presence of 10µl saliva into the *E.coli* and extractant.

ATP detection of *E.coli* in physiological conditions, such as in saliva, confirmed similar results to ***E.coli*** in culture as shown in Figure 4.

The designed luciferase assay was found to detect levels of ATP that correlated with the levels of bacteria in wound exudate. It is evident that there is some degree of product inhibition, and that the enzyme can turn over slowly in the present of ATP and excess luciferin (DeLuca, 1976).

When injecting ATP into the luciferase assay, there is a rapid flash and a very rapid decay to a very low level of luminescence. This rapid flash and decay to the low level, is due to at least two major factors, a) the activation of dehydroluciferin, which is present in the luciferase assay, and acts as a very potent inhibitor of the luciferase or, b) the presence of inorganic pyrophosphate, which destroys the pyrophosphate in the system and thus tends to allow the inhibiting reaction of dehydroluciferin to become much more favoured.

The enzyme in principle can be used to measure total ATP in a sample or it can be coupled to ATP-producing or consuming reactions. One can confirm that higher ATP results in faster decay and therefore greater inhibition. The sensitivity quoted by DeLuca *et al*, (1978) is such that as little as 10^{-14} mol of ATP can be accurately measured. In this study, the minimum ATP detection was found to be 1nM.

Luciferase is ideally stable at -20°C, however, it must not be stored for longer than a couple of days, due to the rapid degradation of luciferase confirmed. The contents, cost, storage temperature and length of storage is summarised in Table 2.

Table 2 Luciferase assay kit

Contents/assay (-20°C for 2 days)	Cost/100 Assays (£)
1ml pH7.8 Luciferase reaction buffer	1.29
10µl 0.9µM Luciferin in pH7.8 spectroscopy buffer	3.83
10µl 32nM Luciferase in pH7.8 Luciferase storage buffer	1.45
10µl Extractant	0.20
Total	**6.77**

Example 3: Use of the Hy-Lite system to determine levels of ATP in 2 bacterial strains

A commercial kit was used, based on an ATP-dependent reaction between Luciferin-Luciferase. This kit is called the Hy-Lite system (Merck Diagnostics, Inc). The kit consist of specific pens to take a sample and a luminometer with a digital readout for displaying relative light units (rlu) emitted from the sample. This kit was used to measure ATP concentrations in different dilutions of various bacterial suspensions.

Materials

20% CS/DMEM is used as a Wound fluid analogue because it has similar protein profile as serum and protein concentration as wound fluid

Calf Serum (CS) from Life Technologies, cat no.16170-078, lot no.37N7765.

Dulbecco's Modified Eagles Medium (DMEM) from Life Technologies, cat no.31885-023.

Bacter Peptone Water (TP) used to dilute bacterial suspension, from prepared as described in DOM M41203: Preparation of media.

Tryptone Soya Agar from Oxoid, cat no. EP0163A

Escherichia coli (E.coli) suspension

Staphylococcus aureus (S.aureus) suspension

30ml plastic universals, printed labels from Bibby Sterilin, cat no. 128C.

Bijou plain label from Bibby Sterilin, cat no. 129B.

The Hy-Lite system (Merck Diagnostics, Inc) containing pens for taking a sample and a luminometer for measuring the relative light units emitted from each sample.

Method

Two bacterial strains were chosen, *E.coli and S.aureus* and grown for 24h @ 37°C.

The cultures were transferred from glass bottles into plastic universals. The samples were centrifuged for 15 minutes at 3000rpm. The supernatant was decanted and the pellet was resuspended in 10ml TP; this was repeated three times.

0.5ml each of the bacterial suspension was added to 9ml of TP and this gave the stock solution which was estimated to be a suspension of 10^{10} bacteria using the Macfarlands Indicator kit. The stock solution was then diluted 1 in 10 to obtain 10^7, 10^6, 10^5, 10^4 and 10^3 suspensions.

The Hy-Lite kit was then used to measure ATP levels in each suspension, as follows;

1. Remove the sampling pen from its packaging, being careful not to touch the sampling stick.

2. Hold the cuvette part of the pen, dip the sampling stick into the diluted suspension of bacteria so that the stick touches the bottom of the universal tube.

3. Remove the sampling stick from the tube after about one second.

4. With the thumb over the button (top of the sampling pen), strike the sampling stick onto the strikepad. This is to force the stick into the cuvette chamber.

5. Align the lugs on the button with those on the pen cap. Firmly press the button so that the contents of the cap are ejected into the cuvette chamber.

6. Holding the cuvette with the blue button uppermost, shake vigorously up and down for at least 10 times. This will mix the sample with the reagents.

Measure the light emitted (relative light units) from the sample using the kit.

Summary of results shown in Figures 5 and 6

Bacterial Suspension	Relative Light Units (RLU) *E. coli*	Relative Light Units (RLU) *S. aureus*
10^3 bacteria	0	0
10^4 bacteria	2000	0
10^5 bacteria	1000	4500
10^6 bacteria	4500	18000
10^7 bacteria	44000	67000

These results are shown in Figures 5 and 6. These graphs illustrate that there is an increase in ATP levels that is proportional to bacterial numbers.

Example 4: Quantification of ATP using a Sigma ATP detection kit

To confirm the results obtained in Example 3, another kit, which also uses enzymatic determination of ATP concentration, was obtained from Sigma. The reaction utilised in this kit is coupled with a dephosphorylation reaction that involves oxidation of NADH to NAD, hence the decrease in absorbance at 340nm can be used to calculate the ATP concentration.

Materials

Wound fluid analogue (20% CS/DMEM)

Calf Serum (CS) from Life Technologies, cat no.16170-078, lot no.37N7765.

Dulbecco's Modified Eagles Medium (DMEM) from Life Technologies, cat no.31885-023.

Bacto Peptone (TP) bacterial suspension medium

Tryptone Soya Agar from Oxoid, cat no. EP0163A

Shaped spreaders from Philip Harris

Escherichia coli suspension

Staphylococcus aureus suspension

30ml plastic universals, printed labels from Bibby Sterilin, cat no. 128C.

Bijou plain label from Bibby Sterilin, cat no. 129B.

Eppendorf tubes from Scientific Laboratory Supplies, cat no.CEN9308

Diagnostic Adenosine –5'-Triphosphate (ATP) Reagents kit from Sigma, cat no. 366-A.

ATP from Sigma, cat no. A-5394

Phosphate buffered Saline (PBS) from Life Technologies, cat no. 14190-094, lot no. 3021885.

Porcine Wound Fluid taken from J&J Medical, Biopolymer Group study. The wound fluid was obtained as follows:

1. Adult Sprague-Dawley rats of approximately 250-300g, with backs shaved, were disinfected using an alcohol swab.

2. Two 0.7 x 0.7 cm full thickness wounds were created on the back of each rat approximately 2 cm away from the spine and half way up the body.

3. The left wound was used as an internal control.

4. The right wound had 1mls of LPS solution at either 0.1 or 1.0 microgram/ml strength injected into the wound edge, 0.25mls in each edge.

5. Each wound was then covered with a 2.0 cm x 2.0 cm Release™ J&J dressing and covered in Bioclusive™ before being wrapped in zinc oxide tape.

6. Rats of both LPS doses were culled at each time point by asphyxiation with carbon dioxide. A 5ml blood sample was retained in lithium heparin tubes for MMP analysis of plasma.

7. Dressings were collected and frozen immediately in pre-labelled 10ml Universal tubes.

8. The wounds were placed in pre-labelled containers containing formalin for histology.

The wound fluid used was pooled from week 1,4,7,10,13,16 and 19 at 72h time point, injected with 1.0 microgram/ml LPS solution. LPS stimulated wounds can be claimed to "imitate" an infected wound as LPS is found on the coat of bacteria and hence should cause a similar response to that of infection.

Method

2 Bacterial strains were grown and dilutions were carried out as described in section 3.3 . ATP levels were measured in each sample using the ATP kit obtained from Sigma. The procedure for using the kit was as follows;

1. Into a 0.3mg NADH vial, pipet in the order indicated;

1.0ml PGA Buffered Solution

1.5ml ddH_2O

0.5ml of a standard or supernatant sample

cap vial and invert several times to dissolve NADH.

2. Decant entire content into a cuvette. Read and record initial absorbance (Initial A) verses ddH_2O as reference at 340nm. Note: Initial A should not be less than 0.6, a lower value may indicate NADH decomposition.

3. Pour content back into vial and pipette 0.04ml GAPD/PGK Enzyme mixture, mix by inversion and read absorbance again.

4. Read and record absorbance verses ddH_2O as reference at 340nm. Continue readings until the minimum absorbance reading is reached (usually requires less than 10 minutes). This is recorded as Final Absorbance (A).

To determine ATP concentration use the following calculation:

ΔA= Initial A - Final A

Sample ATP concentration (μmol/dl) = ΔA* 195

The factor 195 is derived as follows:

195 = (3.04*100)/(6.22*0.25)

3.04 = Volume of liquid in cuvet

100 = Conversion of concentration per ml to concentration per dl.

6.22 = Millimolar absorptivity of NADH at 340nm

0.25 = Sample volume

Standard curve with ATP was made up in both PBS and wound fluid.

Stock of 200μM was made up and diluted down to obtain different concentrations.

ATP Mr = 551.1

To get 200μM Stock = (200*106 * 551.1)/1000 * 100ml = 0.011g

Hence 0.011g in 100ml to get a stock solution of 200μM

Results

Standard curve of ATP in PBS using the kit bought in from Sigma

standard curve	[ATP] microM	delta Abs
	100	0.0980
	50	0.0524
	40	0.0431
	30	0.0336
	20	0.0244
	10	0.0192

ATP measured in *S. aureus* bacterial suspensions, diluted in Wound Fluid Analogue

Bacterial Numbers	Initial A	Final A	Abs 340nm	Conc. (µmol/dl)
10^3	0.8215	0.8188	0.0027	0.5265
10^4	0.8858	0.8757	0.0101	1.9695
10^7	0.9493	0.9357	0.0135	2.6325

ATP measured in *E. coli* bacterial suspensions, diluted in Wound Fluid Analogue

		E.coli		
Bacterial Numbers	Initial A	Final A	Abs 340nm	Conc. (µmol/dl)
10^3	0.8839	0.8788	0.0051	0.9945
10^4	0.889	0.8804	0.0086	1.677
10^7	1.0278	1.0183	0.0095	1.8525

The below standard curve was performed in order to check that the assay still gives a linear response at low levels of ATP.

Standard Curve

PBS

ATP conc microM	I Abs	F Abs	delta Abs
1	0.7971	0.7879	0.0092
2.5	0.8059	0.7961	0.0098
5	0.758	0.7451	0.0129
7.5	0.8405	0.8212	0.0193

PBS

ATP conc microM	Initial Abs	Final Abs	delta Abs
1	0.8053	0.7967	0.0086
2.5	0.7965	0.7864	0.0101
5	0.7914	0.7775	0.0139
7.5	0.8236	0.8063	0.0173
10	0.8361	0.8112	0.0249

ATP conc microM	Delta Absorbance 340nm
0.6	0.0119
1	0.0089
1.6	0.0144
2.5	0.01
5	0.0134
7.5	0.0183
10	0.0249

The following absorbances were recorded in porcine wound fluid using the Sigma assay kit measuring ATP concentration of the order produced by bacterial suspensions present in clinically-infected wounds.

Wound Fluid

ATP conc microM	Initial Abs	Final Abs	Delta Abs
0.6	0.9216	0.9063	0.0153
1.6	0.9687	0.9546	0.0141

Wound Fluid

ATP conc microM	Initial Abs	Final Abs	Delta Abs
0.6	0.9245	0.9161	0.0084
1.6	0.9093	0.8947	0.0146

ATP concentrations in bacterial suspensions were measurable with the Sigma assay (see Figures 7-10). The results obtained in these experiments show that there is a definite increase in levels of ATP with bacterial numbers. The data given above also show that ATP levels that are produced in suspensions containing similar numbers of bacteria to those in clinically-infected wounds, are detectable.

When making a standard curve with ATP and using the ATP kit from Sigma for enzymatic determination of ATP concentration, the numbers differ. This is probably due to interference from the media in which the standard curve is prepared. This however does not affect the results since it is the relative difference in absorbance, which is required for all calculations.

ATP was also measurable in true wound fluid, indicating that there was no interference due to other wound proteins, for example from LPS.

When ATP levels in bacterial suspensions were determined using the kit obtained from Sigma, the apparent ATP level was found not to be proportional to the bacterial numbers, a plateau being observed after an initial proportional rise. It is not clear why this non-linear response occurred, but it may be the outcome of interference between proteases the bacteria produce and the enzyme upon which the assay is based. The plateau is also observed when preparing a standard curve in wound fluid. This could again be due to proteases present, or due to that the upper sensitivity level of the kit is reached.

These results therefore show that there is a proportional increase in ATP levels with numbers of bacteria. The levels of ATP present in a bacterial suspension of a concentration that would be clinically termed an infection, can be detected in wound fluid.

It will be appreciated that modification of detail may be made without departing from the scope of the invention as defined in the accompanying claims.

References

1. Lohmann K: Uber die Pyrophosphatfraktion in Muskel. Naturwissenschaften 17:624, 1929

2. Nahid Turan; Internal Final Year Honours Project: Biochemical Markers of Chronic Wound Processes, Supervisor: Dr R. Chittock, Birmingham University, School of Biochemistry.

CLAIMS

1. A method of monitoring the bacterial contamination of a wound comprising monitoring the adenosine triphosphate (ATP) concentration of wound fluid removed from the wound.

2. The method of claim 1 wherein the wound is a chronic wound.

3. The method of claim 2, wherein the chronic wound is a chronic ulcer such as a dermal ulcer, venous ulcer, pressure sore or decubitis ulcer.

4. The method according to any preceding claim wherein the ATP concentration is measured using an enzyme-coupled reaction.

5. The method of claim 4 wherein the enzyme-coupled reaction is the luciferin-luciferase reaction.

6. The method of claim 4 wherein the enzyme-coupled reaction involves the use of alkaline phosphatase.

7. The method according to either of claims 5 or 6, wherein the enzyme-coupled reaction is monitored using colorimetry, luminescence, chemiluminescence or autoradiography.

8. A wound dressing or biosensor for use in monitoring the bacterial contamination of a wound comprising an enzyme that hydrolyses ATP and a substrate of the enzyme.

9. A wound dressing or biosensor according to claim 8, further comprising a reporter molecule.

10. A diagnostic kit for use in monitoring the bacterial contamination of a wound comprising a wound dressing or biosensor according to either of claims 8 or 9.

11. Use of a wound dressing according to either of claims 8 or 9 for use in the manufacture of a medicament for the diagnosis of bacterial contamination of a wound.

FIG. 1
LUCIFERASE CALIBRATION CURVE AT HIGH CONCENTRATIONS OF ATP

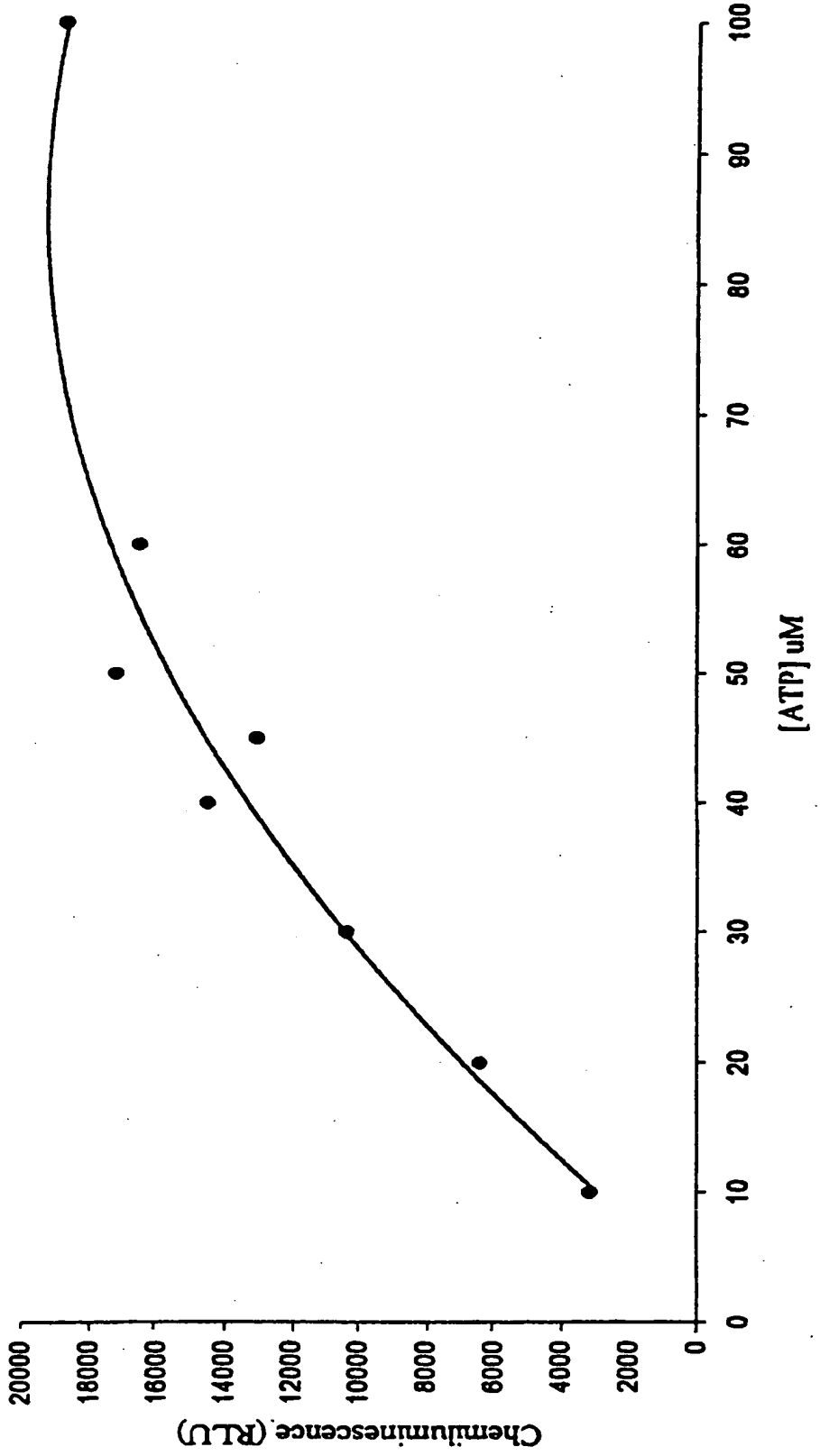


FIG. 2
LUCIFERASE CALIBRATION CURVE SHOWING LOWER ATP CONCENTRATIONS

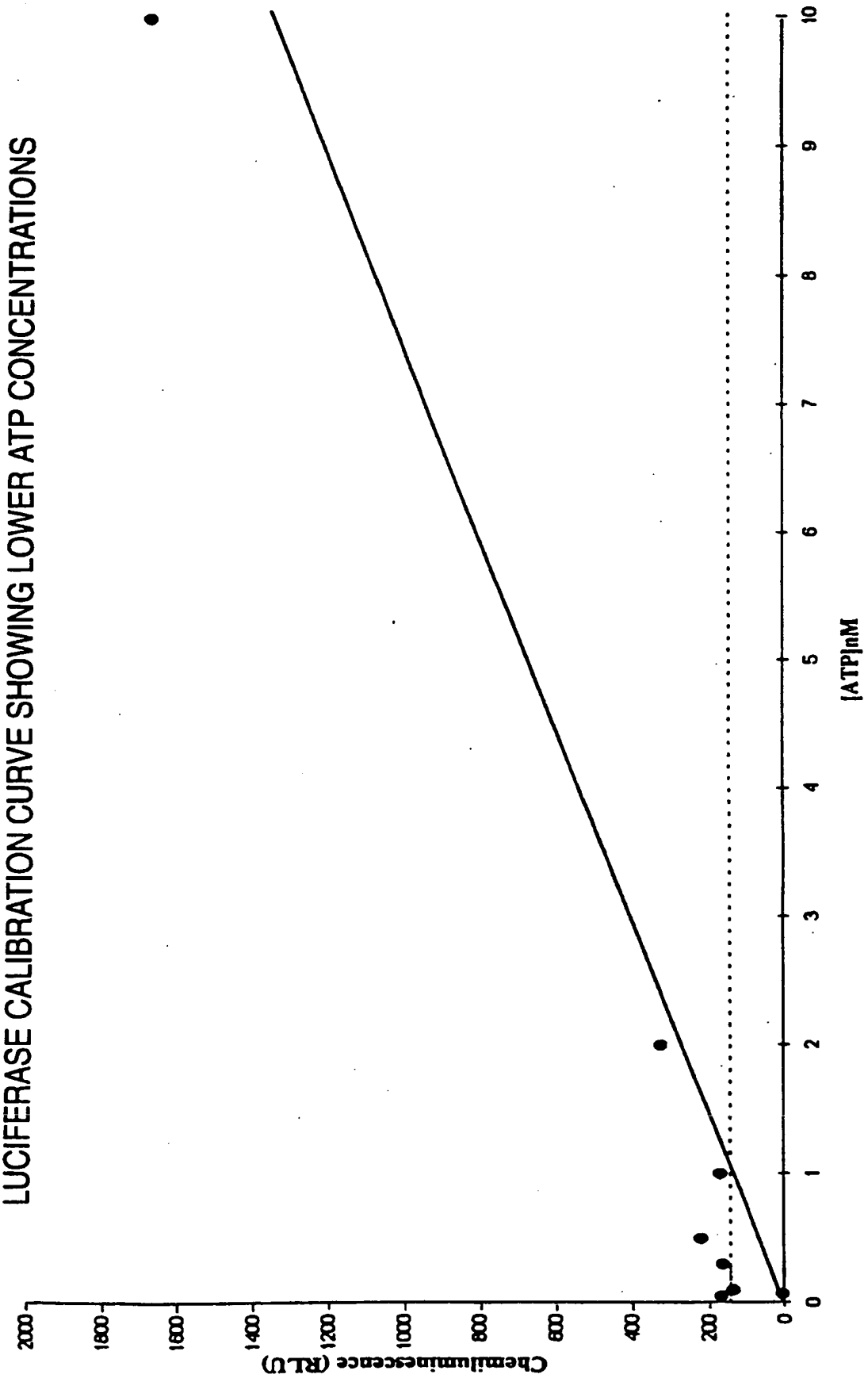


FIG. 3
STABILITY OF LUCIFERASE AT DIFFERENT TEMPERATURES

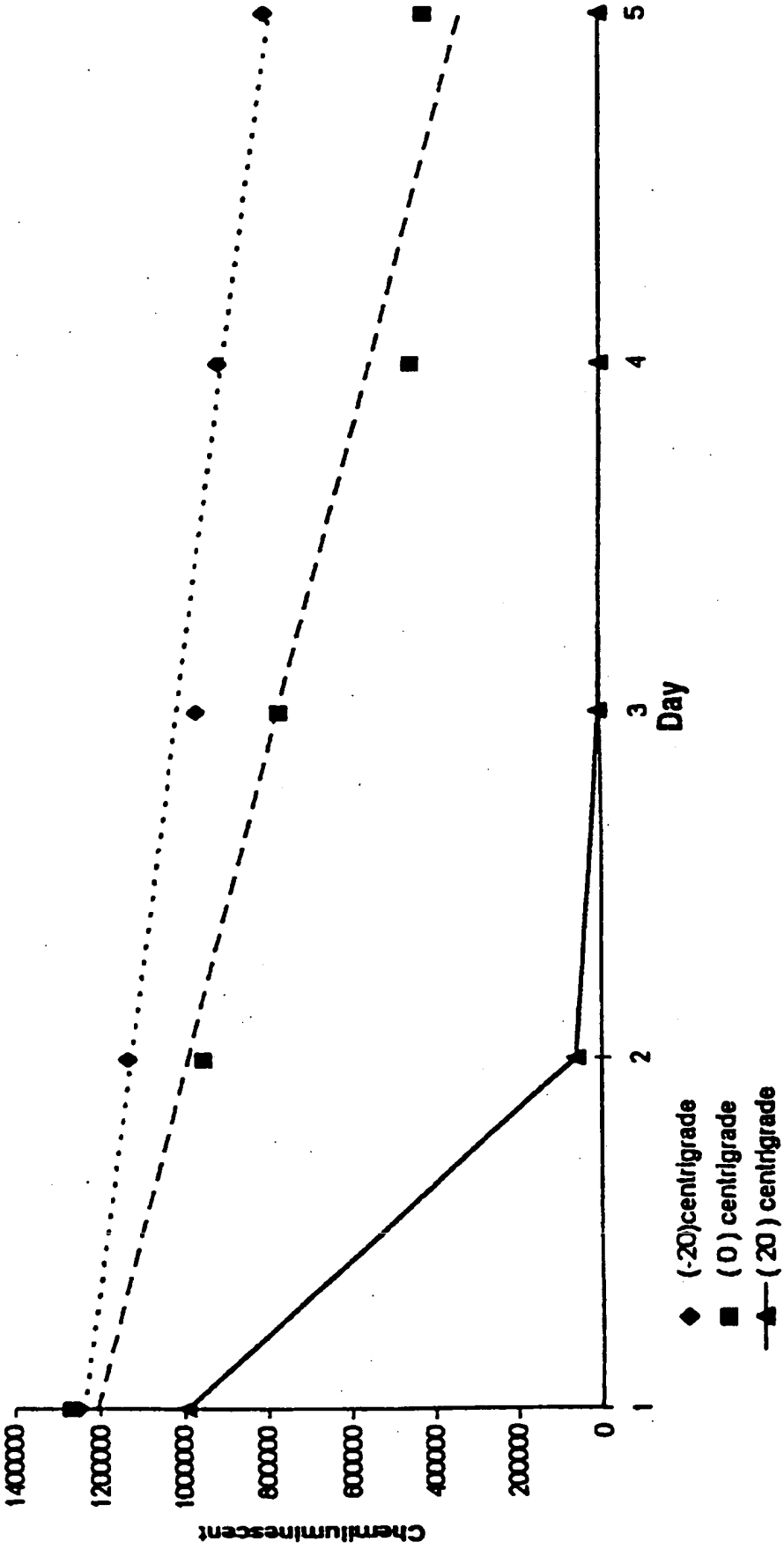


FIG. 4

ATP ACTIVITY AT DIFFERENT NUMBERS OF E.COLI

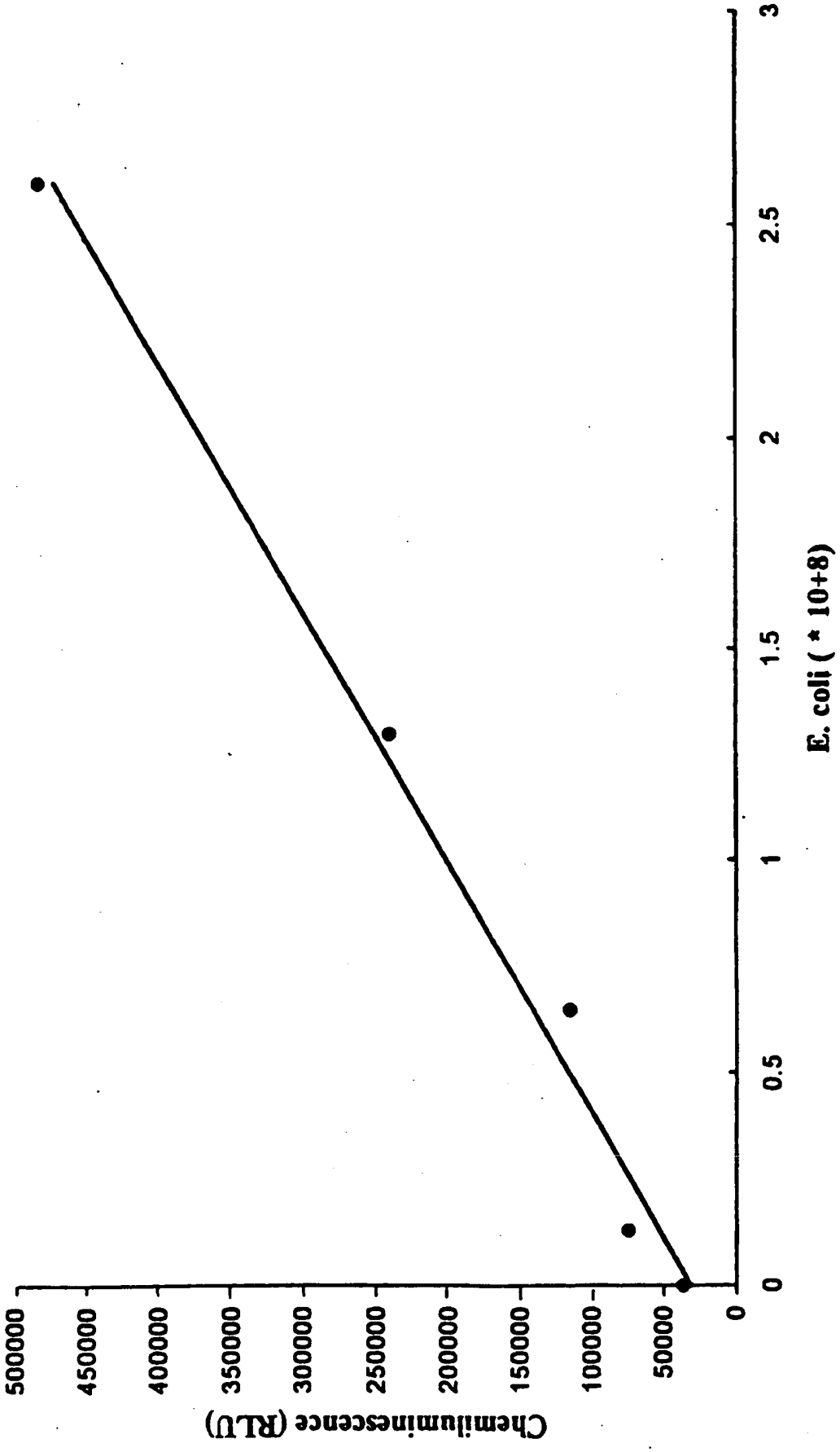


FIG. 5

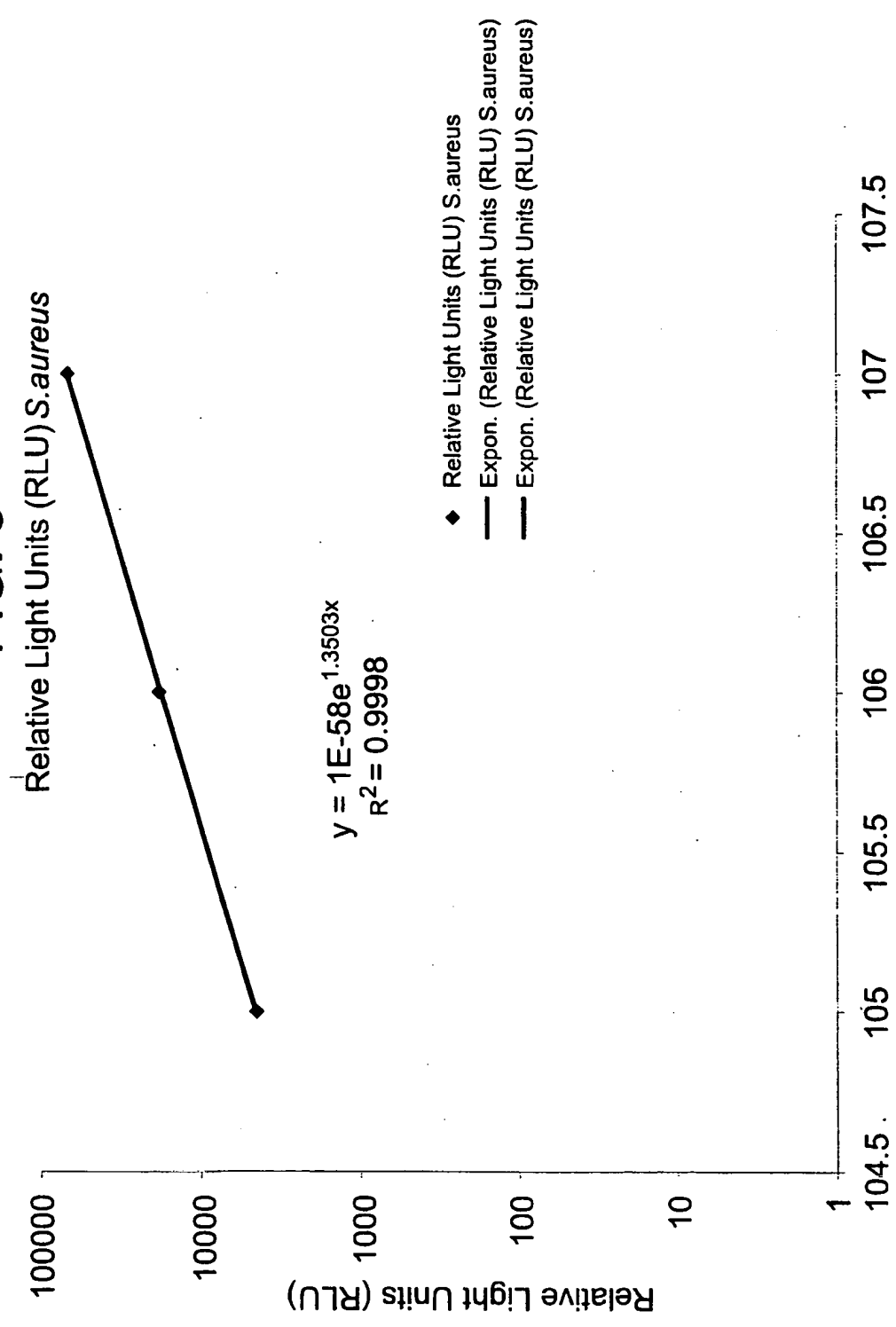

Relative Light Units (RLU) S.aureus
y = 1E-58e^1.3503x
R^2 = 0.9998
Relative Light Units (RLU) S.aureus
Expon. (Relative Light Units (RLU) S.aureus)
Expon. (Relative Light Units (RLU) S.aureus)
Relative Light Units (RLU)
100000
10000
1000
100
10
1
104.5
105
105.5
106
106.5
107
107.5
Bacterial Suspension

FIG. 6

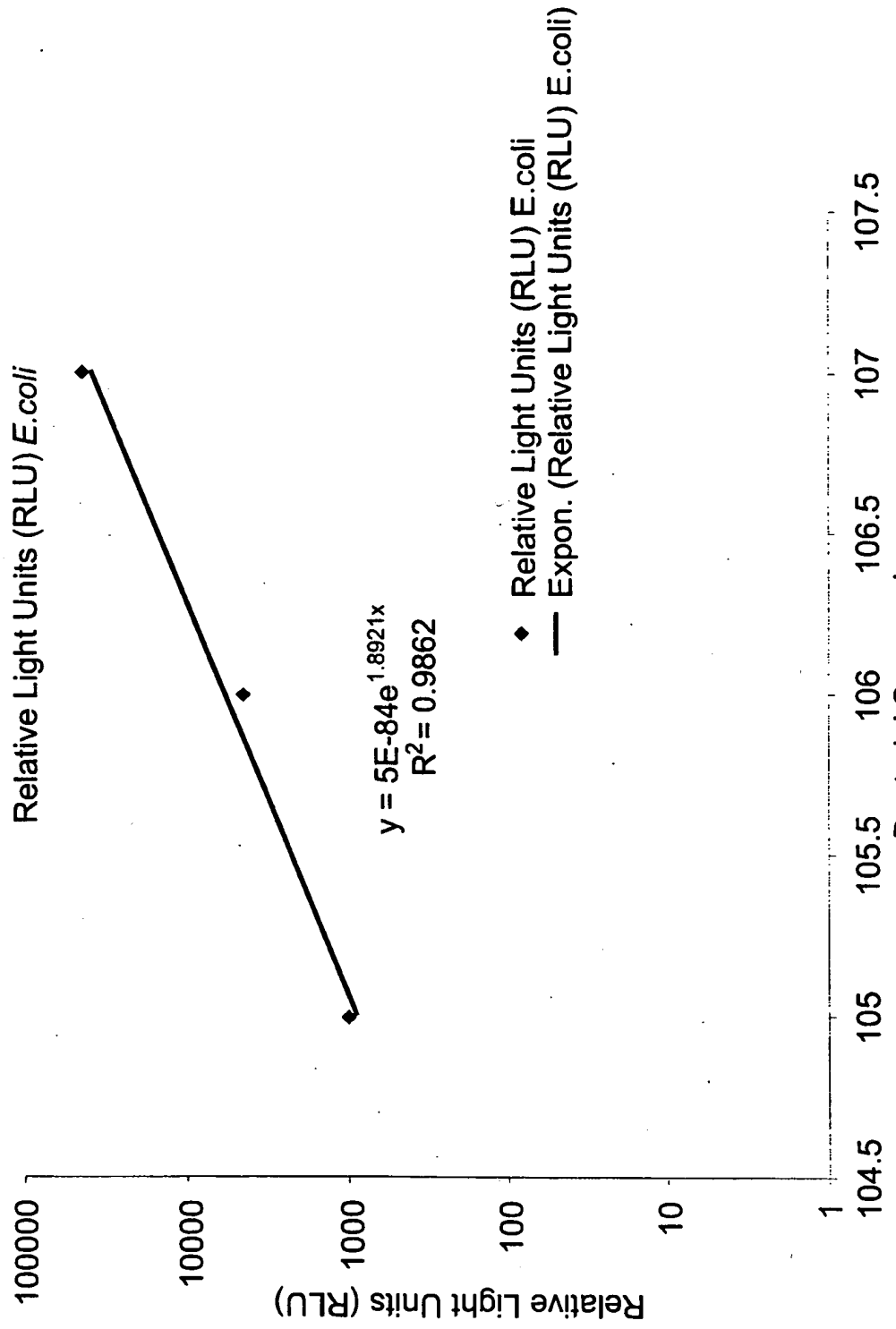

Relative Light Units (RLU) E.coli
y = 5E-84e^1.8921x
R^2 = 0.9862
Relative Light Units (RLU) E.coli
Expon. (Relative Light Units (RLU) E.coli)
Relative Light Units (RLU)
100000
10000
1000
100
10
1
104.5
105
105.5
106
106.5
107
107.5
Bacterial Suspension

FIG. 7

ATP standard curve

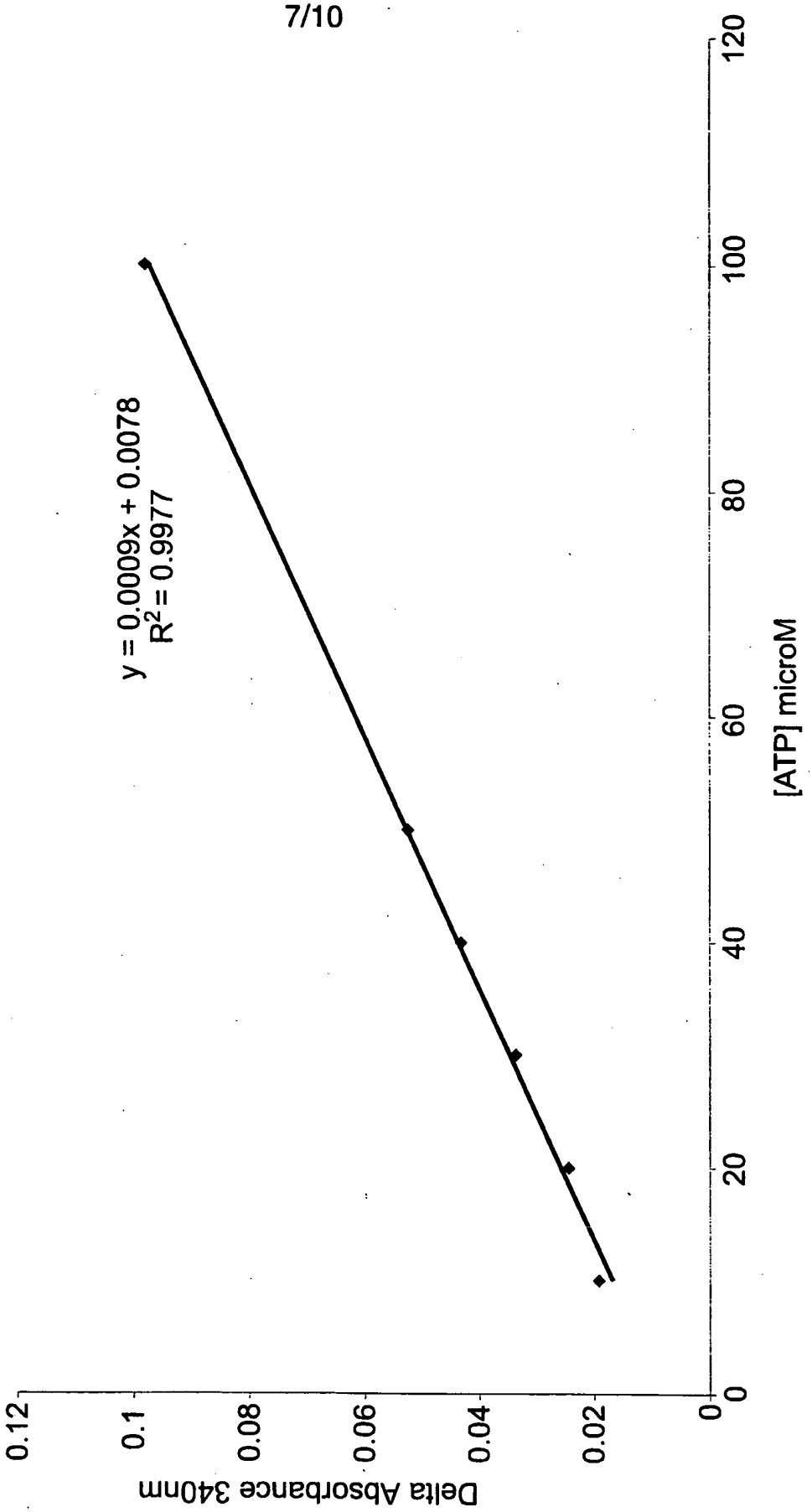


FIG. 8

ATP Concentrations with increasing bacterial numbers, E.coli

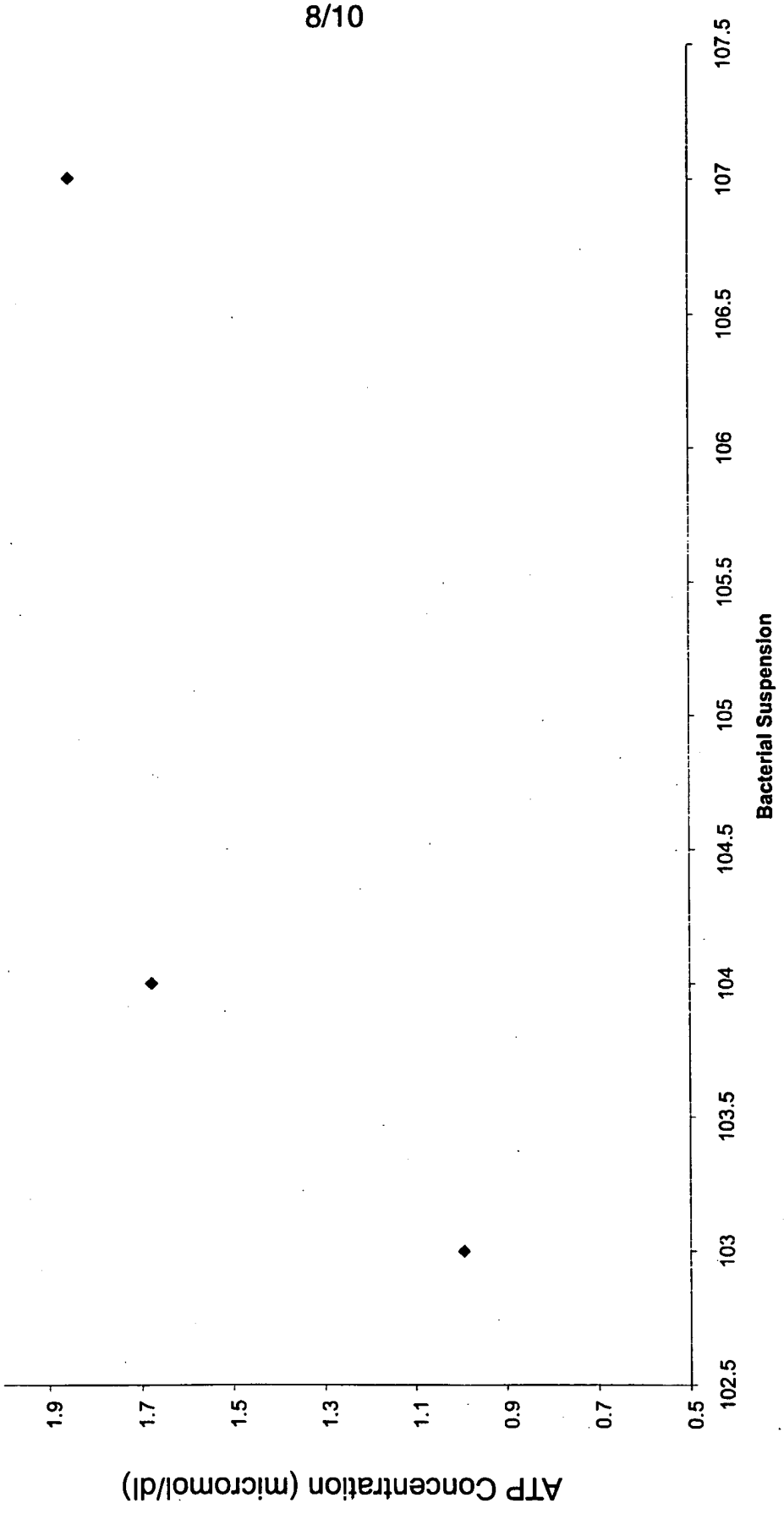


FIG. 9

ATP Concentrations with increasing bacterial numbers, S.aureus

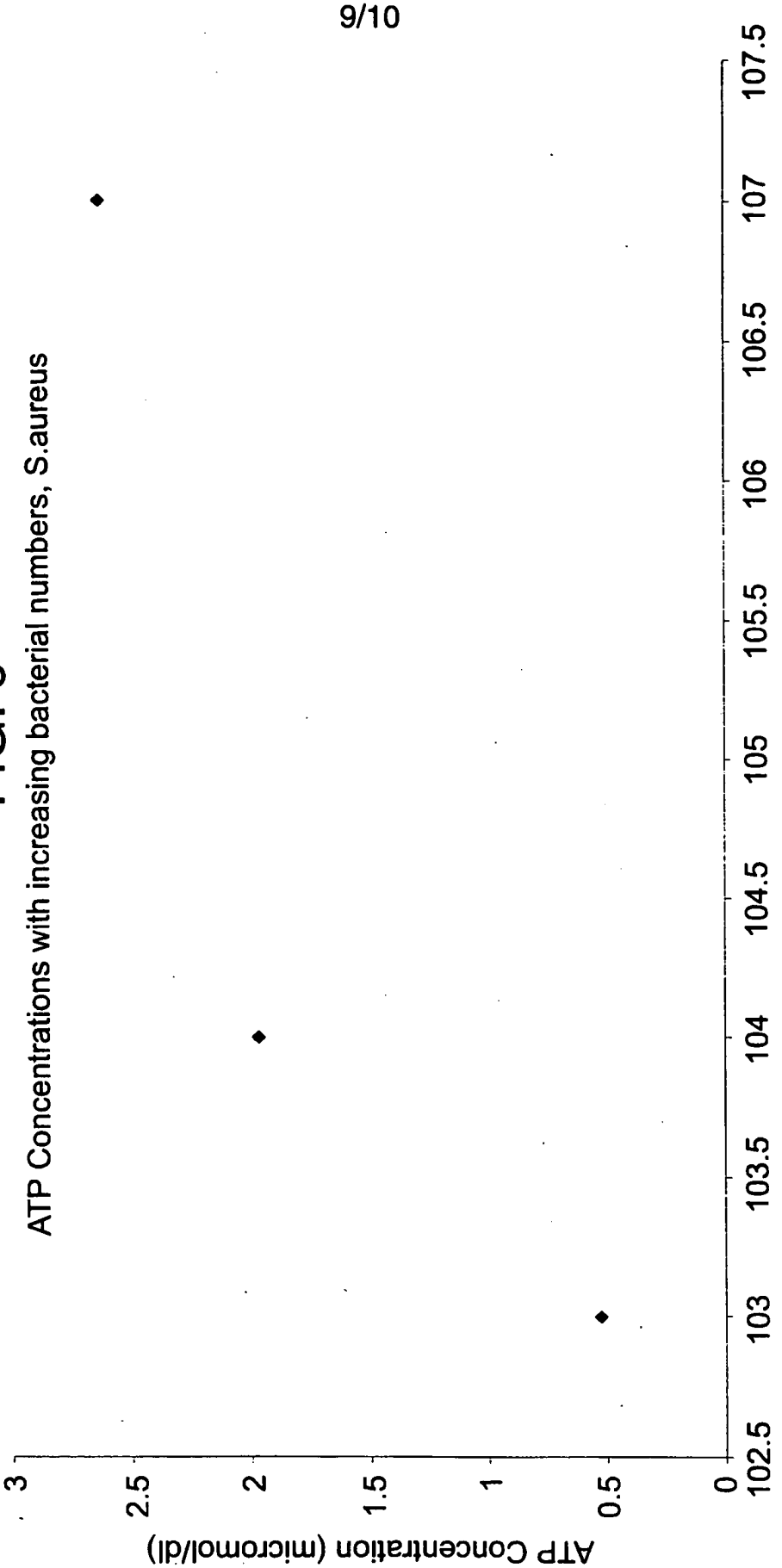
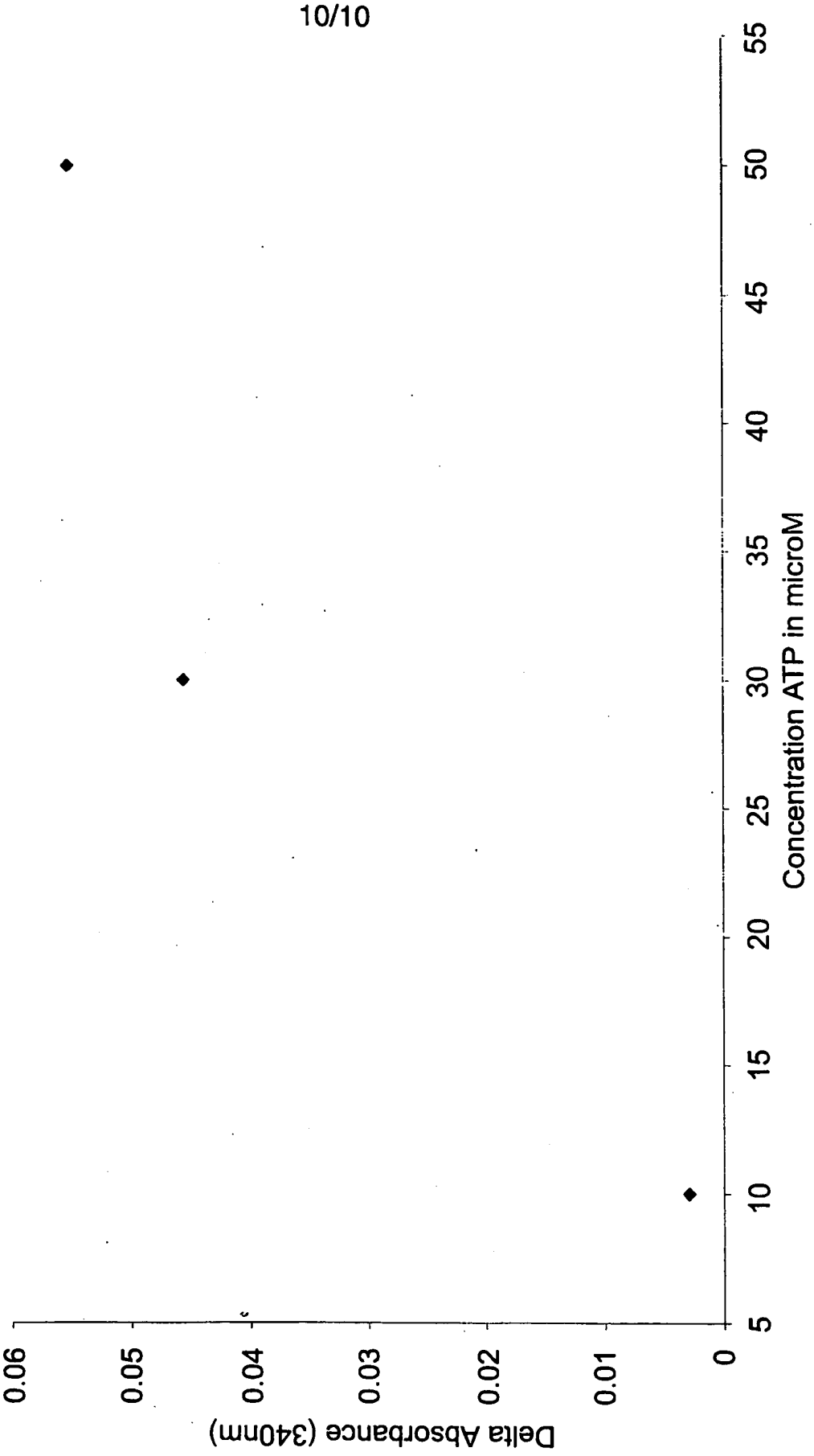


FIG. 10

ATP Standard curve - True Wound Fluid



INTERNATIONAL SEARCH REPORT

International Application No
PC[redacted]99/02585

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/04 C12Q1/66 C12Q1/42 A61L15/38

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q G01N A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 309 429 A (LIFE SCIENCE INTERNATIONAL AB) 29 March 1989 (1989-03-29) page 2, line 9,10 page 4, line 44-49 claims -----	1-11

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search	Date of mailing of the international search report
3 November 1999	12/11/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Pellegrini, P

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter[illegible]ional Application No
PC1/[illegible]9/02585

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0309429 A	29-03-1989	AU 2484988 A	18-04-1989
		SE 8703675 A	24-03-1989
		WO 8902929 A	06-04-1989